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(54) Title: NEW DIAGNOSTIC ASSAY FOR DETECTION OF SYPHILIS

(57) Abstract

This invention relates to a novel assay for the diagnosis of syphilis using human serum or plasma. Syphilis is a sexually transmitted disease by the spirochaete micro-organism *Treponema pallidum*. Antibodies are produced in infected patients. Use of the invention results in a new and efficient means for detecting these antibodies, and hence infection. The invention provides a method for testing for the presence of antibodies to *Treponema* species in blood serum or plasma characterised by the addition of the following components to a reaction vessel in any sequence: a substantially undiluted sample of the test serum or plasma, erythrocytes coated with antigenic components of the target *Treponema* species, and reagents to neutralise the effects of antibodies to non-*Treponema* antigens or antibodies to *Treponema* species other than the target *Treponema* species mixing after the final addition and assessing agglutination of the erythrocytes, wherein the reaction vessel is coated with a binding agent which combats interaction between the vessel surface and the sample and/or erythrocytes causing false positive or false negative agglutination results.

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"New Diagnostic Assay for Detection of Syphilis"

TECHNICAL FIELD

This invention relates to a novel assay for the diagnosis of syphilis using human serum or plasma. Syphilis is a sexually transmitted disease caused by the spirochaete micro-organism *Treponema pallidum*. Antibodies are produced in infected patients. Use of the invention results in a new and efficient means for detecting these antibodies, and hence infection.

BACKGROUND

Although Syphilis has been recognised as a specific disease for over 500 years, it was not until the beginning of this century that the causative organism, *Treponema pallidum*, was first identified. The primary means for detection of the disease was limited to visual identification of the organism in human syphilitic material. Syphilis is transmitted chiefly by direct contact, the organism entering the body through minute pores in the skin or mucous membranes. In practice the most common route of infection is as a sexually transmitted disease. Of particular concern is

that the disease may also be transmitted congenitally from mother to foetus.

There are now available a number of techniques available to diagnose syphilis in the infected individual from a small sample of the patient's blood. The pathogen can be directly visualised once there are sufficient numbers of organisms in the sample, but early diagnosis is hindered by the fact that *T. pallidum* cannot be cultivated *in vitvo* on artificial media. Another common method is to use a non-treponemal test; these tests use cardiolipin as the active ingredient in a mixture with cholesterol and lecithin to detect anti-cardiolipin antibodies found in infected patients. Although providing a rapid method for screening many samples, one of the problems with these non-treponemal tests is the occurrence of both false positives and false negatives.

Of inherently greater specificity are those tests which detect the presence of anti-*T. pallidum* antibodies in the patients blood. These use three main technologies, ELISA, FTA-ABS and haemagglutination. ELISA tests have been developed which use specific antigens bound to a labelling enzyme. These offer good specificity but can be expensive and time-consuming. Another very widely used technique is the fluorescent *Treponema* antibody absorption test (FTA-ABS), in which whole organisms are fixed to glass slides and then overlaid with test serum; the bound antibodies are detected with a fluorescent anti-IgG conjugate. This method is very specific but is laborious to carry out and hence is more generally used as a confirmatory test rather than a primary screen.

Haemagglutination is one of the most commonly used

screening tests. The test uses *Treponema* antigens bound to red blood cells. The presence of anti-*T. pallidum* antibodies in the test serum leads to extensive cross-linking which results in agglutination of the red blood cells to form a visible mat. These are known generally as either *Treponema pallidum* Haemagglutination Assays (TPHA) or Micro-Haemagglutination Assay - *Treponema pallidum* (MHA-TP). These assays are relatively cheap to produce and can be automated by carrying out the reactions in microtitre plates. In a typical TPHA the patients sample is diluted in a suitable diluent and then a proportion of this mixture is added to test cells (avian or ovine erythrocytes coated with *T. pallidum* antigens). The agglutination of the erythrocytes can be seen either with the naked eye or by fully automated optical methods.

One of the inherent problems of prior art TPHAs is the requirement for the dilution step. The need for a dilution step makes the assay harder to automate, increases the time to complete the assay, increases the cost of the procedure and limits the overall sensitivity of the assay. Another disadvantage is the potential loss of integrity of the patient's sample in a two-stage process. The need for a dilution step arises as a result of a number of non-specific binding reactions which can occur between the test sample and the antigens bound to the erythrocytes. The main sources of these non-specific binding reactions are as follows;

1. The *Treponema pallidum* organisms are grown in rabbit testes. Subsequent purification of the antigen is never completely effective with the result that some rabbit antigens co-purify with

the *T. pallidum*. The presence in the test serum of any anti-rabbit antibodies could lead to the development of false positives.

2. The erythrocytes on which the *T. pallidum* antigens are bound have antigenic sites themselves. Any antibodies to these antigens present in the test serum could also lead to the development of false positives.

3. The *T. pallidum* antigens are not completely specific to anti-*T pallidum* antibodies; other commonly found antibodies, particularly those related to commonly found non-pathogenic related species can react, so giving false positives.

A reliable assay for detection of *T pallidum* antibodies must therefore ensure that these three means of interference are overcome, otherwise a significant number of false positives will result. In prior art TPHAs this is generally achieved by the addition of neutralising substances to a diluent. The test sample is then mixed with the diluent so neutralising the various non-specific binding sites. Thus, the rabbit antigens are neutralised by the addition of rabbit serum and the erythrocyte antigens are neutralised using a complex mixture of fragmented red blood cells such as commercially available 'ox stroma'. The reaction of non-*T. pallidum* antibodies with the *T. pallidum* antigens is reduced by adding an autoclaved mixture of related species of *Treponema*. Prior art TPHAs have generally added these neutralising reagents to a diluent. This results in two liquid handling operations; addition of test sample to diluent and the addition of a proportion of the diluent to the activated red blood cells. This dilution step is also

thought to assist in reduction of non-specific binding by diluting out contaminating antibodies.

Prior art attempts to remove the dilution step have involved the addition of all the three neutralising mixtures directly to the activated red blood cells. These attempts have all failed as false positives are not effectively screened out. A second problem is that the positive agglutination patterns formed from genuine positives collapse after a short period. There is therefore a danger of both false positives and false negatives. Therefore, no reliable single stage TPHA for measurement of anti-*T. pallidum* antibodies exists.

STATEMENT OF INVENTION

It is the aim of the present invention to provide a TPHA/MHA-TP which does not require a dilution step, but nevertheless offers a highly specific diagnostic assay.

As used herein the term "substantially undiluted sample" means a sample which is a test serum or plasma in essentially the form in which it is taken from a patient, in contrast to a sample diluted according to the prior art TPHA/MHA-TP practices.

Many TPHAs, when used as a preliminary screen are carried out in small reaction vessels such as microtitre plates. Although automated liquid handling systems can be used with microtitre plates, the requirement for a dilution step adds significantly to the complexity of the assay, with concomitant repercussions on time, cost and sample integrity.

It has now been found that the hitherto insoluble problem of prior art TPHAs, the requirement for a

dilution step, can be overcome by pre-coating the surface of the reaction vessel with a binding agent. This binding agent may react with reactive groups on the surface of the reaction vessel to combat the cross-linking of the sample or erythrocytes to the surface of the reaction vessel leading to partial agglutination (false positives) or interference with true positives (false negatives). Using such pre-coated reaction vessels it has been found that, surprisingly, the neutralising reagents, the activated erythrocytes and the test serum or plasma can be all mixed together directly in the reaction vessel without the false positives and false negatives which have previously been seen.

The present invention thus enables a single stage TPHA assay to be used to routinely screen large numbers of samples which can be readily automated. The removal of the dilution step has the added advantage that the assay can also be made more sensitive, so enabling earlier detection of syphilis.

In one embodiment of the invention a polystyrene microtitre plate is used as the reaction vessel coated with hydrolysed gelatin as the binding agent. Other binding agents which have been found to be effective include foetal calf serum, lactose, bovine serum albumin, rabbit serum and casein digest. It has also been found beneficial to mix certain of these binding agents together, for example a mixture of hydrolysed gelatin and lactose has been found to be a particularly effective binding agent.

In another embodiment of the invention in order to prevent the collapse of high titre positive samples, the reaction vessel is coated with *T. pallidum*.

The invention can be used in a number of different types of reaction vessel made of differing materials. Thus microtitre plates, strip-well plates, cell culture wells, cuvettes, test-tubes and the like can all be coated with binding agent and used to carry out a single stage TPHA assay. These reaction vessels can be made of a number of materials including polystyrene, polypropylene, polyvinyl chloride, polycarbonate, polyethylene, terephthalate G copolymer or glass.

Although TPHAs have generally been carried out using serum samples it has been found that a slight modification allows efficient use of plasma samples. Thus, in another embodiment of the invention it has been found that when using fresh plasma samples the single stage TPHA can be improved by the presence of certain anti-coagulants, particularly heparin; such anti-coagulant will usually be included in the test cell mixture or formulation (the mixture of all other components - coated erythrocytes, neutralising agents etc - to which the test sample is added); its concentration will generally be appreciably greater than that convention for anti-coagulation purposes - eg at least 340 units/ml of test cell formulation.

DETAILED DESCRIPTION OF THE INVENTION

The details of the invention will be described by way of examples.

Example 1 shows the effects of using an uncoated polystyrene microtitre plate in a single stage assay of 20 serum samples found to be negative for *T pallidum* antibodies using a standard two stage TPHA. Of the 20 true negatives only 2 yielded a 'compact button' indicative of a true negative with 1 indeterminate

result. This type of result would be clinically unacceptable for unambiguous diagnosis of syphilis.

Example 2 shows the effect of coating the microtitre plates with a number of different binding agents. Although 2 show improvement over the uncoated plates, hydrolysed gelatin gives the best improvement, increasing the number of 'compact buttons' from 2 to 18, and thus improving the quality of the negative patterns.

Example 3 shows that hydrolysed gelatin, Bovine Serum Albumin (BSA) and Foetal Calf Serum (FCS) all give promising results when mixed with Tween and lactose. All coated plates show an improvement over the uncoated plates.

Example 4 shows that the addition of sugars other than lactose to the protein mixture gives good results, though lactose is marginally better than the other sugars tested.

In Example 5 plasma samples are used in place of the serum samples tested previously. Surprisingly, the results are not as good. This deficiency is readily overcome by the addition of heparin at a concentration of 340 - 1700 units/ml.

Example 6 shows that with a specimen containing a high concentration of anti-*T. pallidum* antibody, collapse of the agglutination pattern is seen unless the plate is coated with *T. pallidum*.

In summary, it has been found that a variety of substances coated onto the reaction vessel prevent the formation of false positives and the collapse of high

titre positives, and so allow a single-stage TPHA to be used as a screening method for both serum and plasma samples. In a preferred embodiment a polystyrene microtitre plate is coated with *T. pallidum*, 2% (w/w) hydrolysed gelatin and 2% (w/w) lactose solution.

EXAMPLES

EXAMPLE 1 UNCOATED MICTROTITRE PLATES

Test Cell Formulation

A formulation of Test Cells is made by mixing together the following reagents:

Chicken erythrocytes coated with *Treponema pallidum* antigen[1]	0.4-0.85% w/w
Bovine serum albumin	5.0 mg/ml
Gentamycin sulphate	20 ug/ml
FTA sorbent	1.9% v/v
Sodium azide	1 mg/ml
Sodium chloride	16 mg/ml
Potassium and Sodium salts	7 mg/ml
Rabbit serum	0.4% v/v
Tween 80	0.1% v/v
Ox stroma	0.01% w/v
Distilled deionised water	

[1]Standard procedures are used to tan and coat the erythrocytes with *T. pallidum* antigen (Tomizawa, T. and Kasamatsu, S. Jap.J.Med.Sci.Biol., 1966,19,305 and Sequiera, P.J.L. and Eldridge, A.E. Brit. J. Vener. Dis., 1973,43,242.)

Specimens

20 fresh (maximum 2 days old) sera previously found to be negative for syphilis antibody for a standard two stage TPHA.

Procedure

10 µl of serum are placed into a well of microtitrate plate. 90 µl of Test Cell Formulation are added and the contents mixed by tapping or shaking the plate. The plate is then incubated for one hour at room temperature, and the plate examined either visually or using an optical instrument.

Interpretation of Results

Agglutination of the cells is interpreted as a positive result. Strong positives may show some folding at the edge at the cell mat. Setting of the cells into a compact button or a button with a pinprick hole in the middle is interpreted as a negative result. Cells showing partial agglutination resulting in a ring with a large hole in the middle are interpreted as a +/- indeterminate reaction.

Results

Agglutination Pattern	Number
Compact button	2
Button with pinprick	17
+/- indeterminate	1
False Postive	0

EXAMPLE 2 - MICROTITRE PLATES COATED WITH BINDING AGENT

A comparison of the effect of different structural types of binding agents bound to polystyrene microtitre plates is shown below.

Each of the binding agents is added to distilled water and sodium azide (0.1%w/v) is fully dissolved and mixed. 200 µl of this solution is added to each well of a polystyrene microtitre plate. The plate is then incubated at 37°C, aspirated and dried. The Test Cell Formulation, Specimens and Procedure are as described in Example 1. The results shown are compared to the uncoated plate in Example 1.

	Binding Agent			
Agglutination Pattern	Uncoated	Hydrolysed gelatin 2% (w/v)	Lactose 2% (w/v)	Tween 20 0.01% (v/v)
Compact button	2	18	16	11
Button with pinprick	17	1	3	7
+/- indeterminate	1	1	1	1
False Positive	0	0	0	1

The results clearly show that two of the three types of binding agent have a beneficial effect. The hydrolysed gelatin has the most significant effect.

EXAMPLE 3 - EFFECT OF DIFFERENT PROTEIN MIXTURES AS BINDING AGENTS

Hydrolysed gelatin is one example of a complex protein

mixture. The data below show the effect of different protein mixtures when combined with a sugar such as lactose (2% (w/v)) and a surfactant such as Tween 20 (0.01% v/v). The Test Cell Formation, Procedure and Specimens are as described in Example 1. The procedure for coating the plates is as described in Example 2.

	Binding Agent				
Agglutination Pattern	Hydrolysed gelatin 2%(w/v)	BSA 1% (w/v)	FCS 2% (v/v)	RS 2% (v/v)	CD 2% (w/v
Compact button	19	19	19	8	11
Button with pinprick	1	1	1	12	9
+/- indeterminate	0	0	0	0	0
False Positive	0	0	0	0	0

BSA - Bovine Serum Albumin
FCS - Foetal Calf Serum
RS - Rabbit Serum
CD - Casein digest

The results show that a number of protein mixtures, when combined with sugar and surfactant, have a beneficial effect over uncoated plates.

EXAMPLE 4 - EFFECT OF ADDITION OF DIFFERENT SUGARS TO HYDROLYSED GELATIN

The effect of adding alternative sugars with hydrolysed gelatin is shown below. The procedure for coating the plates is as described in Example 3, with the lactose being replaced by other sugars.

	Binding Agent			
Agglutination Pattern	Lactose	Maltose	Glucose	Dextran
Compact button	19	18	18	15
Button with pinprick	1	2	2	5
+/- indeterminate	0	0	0	0
Positive	0	0	0	0

The results indicate that the addition of a number of different sugars to a protein such as hydrolysed gelatin has a beneficial effect, with lactose having the greatest effect.

EXAMPLE 5 - EFFECT OF ADDITION OF HEPARIN TO PLASMA SAMPLES

Specimens

20 fresh (maximum 2 days old) citrated plasma samples previously found to be negative for syphilis antibody by standard single stage TPHA.

All of the previous examples have demonstrated the effectiveness of coating the reaction vessel with a binding agent when using a serum sample. To test the effectiveness of the binding agent with plasma samples a polystyrene microtitre plate is coated with hydrolysed gelatin, lactose, Tween 20 and azide as described in Example 3. The results are not as good as with serum. The addition of herapin at a concentration of 340 - 1700 units/ml improves the results, comparable with those achieved for the serum samples.

Agglutination Pattern	Without heparin	With heparin
Compact Button	15	19
Button with pinprick	3	1
+/- indeterminate	1	0
False Positive	1	0

EXAMPLE 6 - EFFECT OF *TREONEMA PALLIDUM* AS A BINDING AGENT

The effect of coating the plate with dilutions of *T. pallidum* prior to coating the plate with hydrolysed gelatin and lactose is shown in the data below. The *T. pallidum* (initial concentration 6.0 x 10^8 organisms/ml) is sonicated, diluted in saline and 100-200 μl is added to each well of a polystyrene microtitre plate. The plate is incubated overnight at 4 C, aspirated and then the procedure for coating the plates is as described in Example 2.

	Concentration of *T. pallidum*				
	0	1/3000	1/2000	1/1000	1/5000
Agglutination pattern of high titre positive	collapse	partial collapse	no collapse	no collapse	no collapse

The results indicate that the addition of *T. pallidum* to the plate coat at a range of dilutions has a beneficial effect.

CLAIMS

1. A method for testing for the presence of antibodies to *Treponema* species in blood serum or plasma characterised by the addition of the following components to a reaction vessel in any sequence:

a substantially undiluted sample of the test serum or plasma,

erythrocytes coated with antigenic components of the target *Treponema* species, and

reagents to neutralise the effects of antibodies to non-*Treponema* antigens or antibodies to *Teponema* species other than the target *Treponema* species

mixing after the final addition and assessing agglutination of the erythrocytes, wherein the reaction vessel is coated with a binding agent which combats interaction between the vessel surface and the sample and/or erythrocytes causing false positive or false negative agglutination results.

2. A method for testing for the presence of antibodies to *Treponema* species in blood serum or plasma which comprises pre-coating a reaction vessel with binding agent which combats interaction between the vessel and the sample and/or coated erythrocytes causing false agglutination results and adding to the reaction vessel in any sequence:-

erythrocytes coated with antigenic components of the target *Treponema* species,

reagents which neutralise the effects of antibodies to non-*Treponema* antigens or antibodies to *Treponema* species other than the target *Treponema* species, and a substantially undiluted sample of the test serum or plasma

mixing after the final addition and assessing the resulting agglutination pattern.

3. A means for testing for the presence of antibodies to *Treponema* species in blood serum or plasma characterised by the addition of the following components to a reaction vessel in any sequence:

a substantially undiluted sample of the test serum or plasma,

erythrocytes coated with antigenic components of the target *Treponema* species, and

reagents to neutralise the effects of antibodies to non-*Treponema* antigens or antibodies to *Treponema* species other than the target *Treponema* species

mixing after the final addition and assessing agglutination of the erythrocytes, wherein the reaction vessel is coated with a binding agent which combats interaction between the vessel surface and the sample and/or erythrocytes causing false positive or false negative agglutination results.

4. A means or method according to any preceding claim in which the binding agent contains at least one component selected from proteins and sugars.

5. A means or method according to Claim 4 in which the binding agent comprises at least one component selected from the group consisting of hydrolysed gelatin, bovine serum albumin, foetal calf serum, rabbit serum, casein digest and lactose.

6. A means for testing for the presence of antibodies to *Treponema* species in blood serum or plasma comprising the addition of the following components to a reaction vessel in any sequence:

a sample of the test serum or plasma,

erythrocytes coated with antigenic components of the target *Treponema* species, and

reagents to neutralise the effects of antibodies to non-*Treponema* antigens or antibodies to *Treponema* species other than the target *Treponema* species

mixing after the final addition and assessing agglutination of the erythrocytes, characterised in that the reaction vessel is coated with a binding agent which comprises at least one component selected from the group consisting of hydrolysed gelatin, bovine serum albumin in combination with a surfactant and a sugar, foetal calf serum, rabbit serum, casein digest and lactose.

7. A means or method according to any preceding claim in which the binding agent comprises bovine serum albumin in combination with a surfactant and a sugar.

8. A means or method according to any preceding claim in which the binding agent comprises bovine serum

albumin in combination with TWEEN® and a sugar.

9. A means or method according to any preceding claim in which the binding agent comprises bovine serum albumin in combination with TWEEN® and lactose.

10. A means or method according to Claims 4, 5, 6 or 7 in which the binding agent comprises both hydrolysed gelatin and lactose.

11. A method for testing for the presence of antibodies to *Treponema* species in blood serum or plasma which comprises pre-coating a reaction vessel with binding agent which comprises at least one component selected from the group consisting of hydrolysed gelatin, bovine serum albumin in combination with a surfactant and a sugar, foetal calf serum, rabbit serum, casein digest and lactose and then adding to the reaction vessel in any sequence:

erythrocytes coated with antigenic components of the target *Treponema* species,

reagents with neutralise the effects of antibodies to non-*Treponema* antigens or antibodies to *Treponema* species other than the target *Treponema* species, and

a sample of the test serum or plasma

mixing after the final addition and assessing the resulting agglutination pattern.

12. A diagnostic test kit for testing for the presence of antibodies to *Treponema* species in blood serum or plasma, the kit comprising the following components

erythrocytes coated with antigenic components of a target *Treponema* species,

reagents to neutralise the effects of antibodies to non-*Treponema* antigens or antibodies to *Treponema* species other than the target *Treponema* species, and

a reaction vessel

and wherein the reaction vessel is coated with binding agent which combats interaction between the vessel and one or both of [a] serum or plasma and [b] the coated erythrocytes which would distort haemagglutination assessment, wherein the binding agent is at least one component selected from hydrolysed gelatin, bovine serum albumin in combination with a surfactant and a sugar, foetal calf serum, rabbit serum, casein digest and lactose.

13. A diagnostic kit according to Claim 12 in which the binding agent comprises bovine serum albumin in combination with TWEEN® and a sugar.

14. A diagnostic kit according to Claims 12 or 13 in which the binding agent comprises bovine serum albumin in combination with TWEEN® and lactose.

15. A diagnostic kit according to Claim 12 in which the binding agent comprises both hydrolysed gelatin and lactose.

16. A means, diagnostic kit or method according to any preceding claim in which the reaction vessel is a microtitre plate, a strip-well plate, a cell culture well, a test-tube or a cuvette.

17. A means, diagnostic kit or method according to any preceding claim in which the reaction vessel is made of polystyrene, polypropylene, polyvinyl chloride, polycarbonate, polyethylene terepthalate G copolymer or glass.

18. A means, diagnostic kit or method according to any preceding claim in which the reaction vessel is a polystyrene microtitre plate.

19. A means, diagnostic kit or method according to any preceding claim in which the target *Treponema* species is *Treponema pallidum*.

20. A means or method according to any preceding claim in which the test sample is blood plasma and the addition to the reaction vessel includes heparin.

21. A diagnostic kit according to any preceding claim in which the components include heparin.

22. A means, diagnostic kit or method according to Claims 20 or 21 wherein the heparin concentration is at least 340 units/ml of the test cell formulation before admixture with a test sample.

23. A means, diagnostic kit or method according to any preceding claim in which *T. pallidum* is present as binding agent.

24. A means, diagnostic kit or method according to any preceding claim in which *T. pallidum*, hydrolysed gelatin and lactose are present as binding agent.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/571 G01N33/555

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 7720, Derwent Publications Ltd., London, GB; Class B04, AN 77-35388Y & JP,A,52 044 229 (FUJIZOKI PHARM KK) 7 April 1977 see abstract ---	1-24
A	DATABASE WPI Section Ch, Week 9145, Derwent Publications Ltd., London, GB; Class B04, AN 91-328419 & JP,A,3 218 465 (SEKISUI CHEM IND KK) 26 September 1991 see abstract --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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